PLANT GROWTH REGULATORS MEDIATED CONSEQUENCES ON MORPHO-PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERSTICS OF STEVIA REBAUDIANA BERTONI

SYNOPSIS

Submitted in partial fulfillment of the
requirements for the award of the degree of
DOCTOR OF PHILOSOPHY
in
Botany
Submitted by

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Enrollment No.: H14844

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2022-2023

INTRODUCTION

Since ancient times, plants have been used in medicine and are still used today (Grover et al., 2002; Chattopadhyay et al., 2007). In the Charak Samhita, around four hundred to five hundred medicinal plants are included (Jain et al., 2018). Medicinal plants are the natural resources in developing of new drugs and are serving as raw material for drugs which are effective in health care for people. Every part of plant such as flower, root, and stem, leaf, fruit, seed and whole plant has medicinal properties. The plant parts are used in different forms. Several herbal medicines are being derived from plants that are used for curing various human diseases (Modak et al., 2007; Shakya & Shukla, 2011). Across the world, rural people rely on traditional local knowledge of medicinal plants for primary health care (WHO, 2019). According to World Health Organization (1991), about 80% of the world population relies on traditional medicine, which is based on plant material. Medicinal plants and herbal medicines form an important component of treatment in the indigenous systems that have evolved in different cultures over several centuries. A number of scientific studies on medicinal plants have indicated that essential phytochemicals can be developed for many health problems (Mishra et al., 2001). Plants produce different bioactive molecules, making them a rich source of different types of medicines that have helped to cure viral infections or pandemics like COVID-19 (Murugesan et al., 2021).

Globally, 17-18% of world flora is utilized for medicinal purpose (Schippmann et a1., 2006; Alamgir, 2017). China and India have the highest numbers of medicinal plants used, with 11,146 and 7500 species, respectively (Srujana et al., 2012; Kopaei, 2013). Nature has bestowed our country with an enormous wealth of medicinal plants; therefore, India has often been referred to as the Medicinal Garden of the world. In India, the majority of the people of tribal and rural areas near the Himalayan ranges depend on their traditional knowledge to combat various diseases and infections by using local medicinal plants, herbs, or shrubs. India is bestowed with a gift of over 45000 plant species (approx 20% of global spp) of which 35000 species of both higher and lower plant groups are of medicinal value (Shipla et al., 2015). The northern part of India possesses a great diversity of medicinal plants because ofthe royal Himalayan range. In India, approximately 75% of the population lives in rural areas (Chintakula, 2020). Himachal Pradesh is the top repositories of medicinal herb (Badola & Pal, 2003). Medicinal plants of Himachal Pradesh are found in the subtropical mountains and low hills below 700 m above mean sea level. Rural people have inherited traditional medicinal plant knowledge from generation to generation.

Several medical problems arise due to more intake of sucrose content which is associated with

negative effect on human body such as obesity, high blood pressure, atherosclerosis, coronary heart disease, high cholesterol and insomnia (Anton et al., 2010; Puri et al., 2012). These problems are correlated with easy and excess availability of high calorie food and beverages. Therefore, for more than two decades, there has been a very strong increase in interest in sweeteners, which do not introduce additional calory into the organism (Brahmachari et al., 2011; Pecivova et al., 2013). Sweetening agents are often used in the production of non-alcoholic beverages (Mc Quillan et al., 1995; Hernandez et al., 2018), confectionery products (Hartel, 2010), pickles, sauces (Bright, 1999), foodstuffs (Kroyer, 2010) and dietary supplements (Ayenew et al., 2009). Consumption of added sugars has negatively contributed to human health (Hernandez et al., 2018; Brown et al., 2010; Grembecka, 2015). Due to increase in the number of patients of obesity (Ludwig, 2018; James, 2008) and diabetes (Wilson, 2010; Ghouri et al., 2017), it is important to educate consumers for making healthy food choices.

Stevia rebaudiana Bertoni is a perennial herb of the Asteraceae family. It is commonly known as sweet leaf, honey yerba, honeyleaf, meethi tulsi, sweet chrysanthemum and candy leaf (Carakostas et al., 2008).It is native to South America (Singh & Rao, 2005; Reis et al., 2017). It is grown commercially in many parts of Brazil, Paraguay, Central America, Thailand, Korea, China and India. Rajasthan, Maharashtra, Kerala and Haryana are the main stevia growing states in India. The most common species of genus Stevia with sweetening potential are *S. dianthoidea, S. phlebophylla, S. ani-sostemma, S. bertholdii, S. crenata, S. enigmatica, S.eupatoria, S. lemmonii, S. micrantha, S. plummerae, S.rebaudiana, S. salicifolia, S. serrata* and *S. viscid* but, among these only *Stevia rebaudiana* exhibits the highest level of sweetness (Carakostas et al., 2008). *Stevia rebaudiana* Bertoni is a natural sweetener plant. Climatic conditions play important roles on the growth and secondary metabolites of stevia plant (Pal et al., 2015). It grows well upto optimum growth under semitropical, subhumid climatic conditions with average annual temperature of 25°C and rainfall of 1375 mm (Gupta et al., 2013). Stevia prefers a well-drained fertile and loam or loam soil high in organic matter and also prefer lighter acidic to neutral (pH 6-7) soil for better growth. Long spring and summer days favor leaf yields and leaf stevioside contents (Martini et al., 2016).

The chemical compound obtained from stevia is considered to be the best alternative source of sugar especially for diabetic patients. Raut & Aruna (2017) reported that the active constituents that are mainly present in *Stevia rebaudiana* are Steviol glycosides that are 150-300 times sweeter than sugar. Steviol glycosides are plant secondary metabolites belonging to a class of chemical compounds known as diterpenes. It is mainly used as a sweetener and

flavor enhancer in the food and beverage industry. The leaves of stevia are the source of compounds called steviol glycosides, viz., stevioside, rebaudioside – A, B, C, D, E and dulcoside-A. Its sweetness is due to ent-kaurene-type diterpene steviol glycosides (SGs), such as stevioside (Ste) and rebaudioside-A (Reb A) (Koheda et al., 1976; Kennelly, 2002; Martini et al., 2017). These steviol glycosides are between 4 to 20% of leaf dry weight (Yadav et al., 2011; Lemus-Mondaca et al., 2012). Apart from *S. rebaudiana*, steviol glycosides have been found in three other species: *Stevia phlebophylla* (Kinghorn et al., 1984), *Rubus suavissimus* (Tanaka et al., 1981; Ohtani et al., 1992; Uhler and Yang 2018) and *Angelica keiskei* (Zhou et al., 2012). However, steviol glycosides content in these plants is rather low, so they are of less economic importance than *S. rebaudiana*. The amount of chemical components varied at different percent level such as diterpene glycosides range from4 to 20% stevioside, 4 to 13% of dry weight, rebaudioside A 2-4%, rebaudioside C 1-2% and dulcoside A 0.4- 0.7% (Gasmalla et al., 2014; Pol et al., 2007). Method of drying and processing also affects the chemical composition of stevia active constituents (Gasmalla et al., 2014). Bergs et al. (2012) suggested that stevioside (Ste) and rebaudioside A (Reb A) are the primary glycosides of stevia plants which contributes 90% weight of all sweet glycosides present in the leaves.

Among all SGs in stevia leaves, Reb-A is the most favorable sweetener. It is 300 times sweeter than sucrose (Joseph & George, 2019). Even Stevioside, the most abundant SG in stevia leaves, has lagged behind Reb-A in the global market, since it has higher water solubility and a more pleasant taste with no bitter aftertaste (Kennelly, 2002). The stevioside contains steviol, an aglycone glycoside (Geuns, 2003) which is thermostatic, pH stable, and non-fermented; hence, it can be used easily in cooked food and drinks without any quality loss (Abdul lateef & Osman, 2011). Reb A was the first commercial steviol glycoside launched in the marketplace (Ashwell, 2015). Steviol glycosides have the potential to outcompete with artificial sweeteners because of their unique features, e.g., low-caloric, non-cariogenic, non-geno-toxic, non-allergic, anti hyperglycemic, antioxidant, antimicrobial, anti-fungal activities, and high water solubility, as well as medicinal properties such as anti-inflammatory and antitumor effects (Kennelly, 2002; Lemus-Mondaca et al., 2012). Labdane Sclareol a compound present in the leaves of stevia has anti-tumorous and cytotoxic properties (Kaushik et al., 2010; Escutia-López, et al., 2019).

Stevia has attracted economic and research interests mainly due to sweet and therapeutic properties of leaves. In India, stevia has generated interest among plant biotechnologists and pharmacologists in terms of its industrial or commercial and pharmaceutical importance (Debnath, 2008; Abdul Razak et al., 2014; Bisht et al., 2017). As regard to diabetic market in

India, stevia is giving good remuneration to the farmers (Hossain et al., 2017). In the coming years the production of stevia leaves will overcome the demand and supply problem but there is need to explore the new areas for successful production of the leaves (Oviedo-Pereira et al. 2015). Therefore, the production of *S. rebaudiana* varieties with a higher Reb-A/Ste ratio is of great importance (Yadav et al., 2011; Yücesan et al., 2016a). In northern India, the plant was introduced during 2000 by Institute of Himalayan Bioresource Technology, Council of Scientific Industrial Research (CSIR), Palampur (Megeji et al., 2005). Now stevia is grown in many parts of India.

Many factors including age, seasonal variation, nutrition, temperature and phytohormones have an influence on accumulation and metabolism of secondary metabolites (Sangwan et al., 2001). There are numerous reports in the literature that concerns with the effects of growth regulators on growth and development of various plants (Sangwan et al., 2001). Most of these growth substances have exhibited influences by modifying the growth characters. Plant hormones, also called phytohormones or plant growth regulators are organic molecules which affect the physiological processes of plant, including differentiation, development, and growth, at low concentrations (Davies, 2004). Plant hormones are involved in various processes including plant growth and development, cell division, cell elongation and differentiation, and apical dominance (Davies, 2013). The most relevant role of hormones is related to the control and cordination of cell division, growth and differentiation (Miransari & Smith, 2014). Plant growth regulators play vital role in the plant developmental processes (Asgher et al., 2015). Auxins, gibberellic acid, abcisic acid, and ethylene are the five main classes of plant hormones and are known as the signal molecules required in many plant development processes (Wu et al., 2009; Jamil et al., 2012; Robles et al., 2012). Among all plant growth regulators, auxin and gibberellins play vital role in regulating developmental processes in plant (Gou et al., 2010).

Auxins are the primary plant growth regulator that functions virtually in every perspective of plant growth and development (Davies, 2004; Weijers & Wagner, 2016). These are the most important group of plant hormones that affect plant growth and development either alone or in combination with other plant hormones (Miransari et al., 2014). They promote cell elongation, especially of shoots, and induces apical dominance and rooting. It control root growth, root gravitropism, and vascular differentiation (Aloni et al., 2006) and also constitute other major groups of plant hormones. Auxins are well known for their role in cell elongation and differentiation in the plants. These are primarily well known for their ability to induce cell elongation and differentiation in the plants. Auxins are a class of phytohormones that control

numerous processes of plant growth and development (Davies, 2013). They have numerous physiological impacts on the medicinal and aromatic plants included cellular elongation and differentiation, apical dominance, root initiation and fruit development (Taiz & Zeiger, 2010, Khan et al., 2015). In addition, they stimulate cell division, vascular differentiation, and root initiation. One of the most common auxins produced by plants is indole-3-acetic acid (IAA), which is the first identified plant hormone (Fahad et al., 2015; Waheed et al., 2015). Indole-3-acetic acid (IAA) is an essential auxin in the plant, which is produced mainly in meristematic tissues of young leaves (Kaya, et al., 2010). It is the most active form of auxin in most plants (Raza et al., 2014). Only low doses of auxin are effective in growth promotion (Vwioko & Longe 2009).

Gibberellins are endogenous plant growth regulators, which improve the growth of plants. In many plants, these active constituents are generally present in the leaves or flowers. Gibberellins have been used to improve growth and herb yield of aerial parts of the plants. Gibberellin helps in cell growth of stem, leaves and other aerial parts by causing cell elongation, and increase in inter nodal length. Gibberellic acid increases the growth of root, shoot and number of leaves by altering the process of cell division and cell elongation (Abdel-Hamid, 2014). It increases the biosynthesis of several secondary metabolites like steroids, terpenoids, flavonoids, and anthocyanins (Srivastava & Srivastava, 2007; Sharma & Kumar, 2011). Commercially, the most common gibberellin is GA_3 (Gibberellic acid), and it is used to increase the length of the plant and to enhance plant yield (Kumlay & Eryigit, 2011). When applied at low concentrations, GA_3 uplifts the seed dormancy, increases growth and overall plant productivity (Binenbaum, 2018). Since 1930s, plant growth regulators have been investigated for their function in agricultural products (Kumlay & Eryiğit, 2011). Both these hormones have been used for improvement of growth and development of aromatic plants.

Nowadays, people are becoming more and more health and diet conscious, so sugar is being replaced by low- calorie sweeteners. Artificial sweetening agents are generally used in the preparation of Sugar-free Syrup. Sweetening agents are employed in liquid formulations designed for oral administration specifically to increase the palatability of the therapeutic agent. Artificial sweeteners have been developed as sugar substitutes (Chattopadhyay et al., 2014).These are included in many “sweets” and are anticipated to be a “sugar” substitute in patients with diabetes mellitus (Evert et al., 2019). Until the 1980s there were only 3 types of artificial sweeteners available: saccharin, cyclamate and aspartame, known as the first-generation sweeteners (Nofre & Tinti, 2000) Saccharin was the first artificial sweetener, which started being marketed in the US in 1901. Since then, a second generation of

sweeteners, represented by sucralose and acesulfame-K, has been approved for human consumption and are gradually conquering the market worldwide (Cabral et al., 2018). Some natural sugar substitutes are known, including Sorbitol and xylitol, which are found in berries, fruit, vegetables, and mushrooms (Ansari et al., 2015). Artificial sweeteners are also called non-nutritive sweeteners because they have very little caloric value. Consumption of added sugars has been implicated in an increased risk of a variety of diseases. Common artificial sweeteners include aspartame, saccharine, sucralose, neotame and acesulfame potassium. Moreover, artificial sweeteners have side effects in terms of obesity, cardiovascular disease, and mortality (Mossavar-Rahmani et al., 2019; Debras et al., 2022; Debras et al., 2022 &Yan et al., 2022). It has also been reported that synthetic sweeteners like aspartame, cyclamate, neotame, etc. are less stable as compared to Stevioside (Rajasekaran et al., 2008).

Stevia and its products are used in food groups such as carbonated beverages, non- alcoholic beverages, table-top sweetener formulations, chewing gum and yogurt etc. It is commercially used in preparation of pickle, vegetables, dried seafood, soya sauces and miso products (Tadhani & Subhash 2006; Goyal et al., 2010). Global food industry has recognized the importance of high-purity zero calorie natural stevia extract because its natural source appeals to many consumers. Truvia, Pure Via and Sun crystals are the major international brands uses purified stevia leaf extract to develop natural table-top sweetener which keeps the calories down in a natural way and enhance the sweetness (Singh et al., 2019). Hence, there is an urgent need to substitute sugar and artificial sweetners with natural low-calorie sweeteners like *Stevia rebaudiana* which will increase the nutritional profile of the products. Therefore, the production of *Stevia rebaudiana* with a higher Rebaudioside - A and Stevioside ratio under the influence of phytohormones is of great importance (Yadav et al., 2011; Yücesan et al., 2016a).

LITERATURE REVIEW

Morphology: Traditionally, morphology of a plant deals with the study of the form and structure including root length, shoot length, leaf area, number of leaves etc. The influence of various external and internal factors alters the morphology of plants directly or indirectly by showing positive and negative effect on morphological features of the plant.

Root length: Root elongation is iterative processes in root development (Atkinson et al., 2014). Auxin controls growth and morphology of roots (Rao & Ravishankar, 2002). Many of the studies showed the effectiveness of different concentrations of GA_3 and IAA on the different plants. Bais et al. (2001) reported that the exogenous treatment of GA_3 induced an

increase in root growth in the hairy roots of *Cichorium intybus* L. Ngatia et al. (2004) noticed increased root length due to application of GA_3 in *Phaseolus vulgaris*. Aygun and Dumanoglu, (2006) stated that root length enhanced in *Cydonia oblonga* Mill by applying GA_3 exogenously on the leaves. 390mg $L^{-1}$of GA_3 application significantly promoted the elongation of the root of *Spondias tuberosa* plants (Santosmatos et al., 2020). Exogenous application of IAA resulted an increased initiation in roots of *Oryza sativa* L. (Chhun et al., 2003). Stefancic et al. (2005) have resulted from his studies that IAA positively increased the length of roots in *Prunus* "GiSelA5". Ozel et al. (2006) analyzed the effectiveness of IAA in *Centaurea tchihatcheffii* and found an enhanced root length as compared to untreated plants. Exogenous application of IAA has been reported to increase the growth of the root of wheat seedlings (Egamberdieva, 2009). Hussain et al. (2011) evaluated that root length in *Cassia absus* improved by increasing the concentrations of IAA.

Shoot length: It has been observed from various findings that treatment with GA3 and IAA resulted the positive effect on the various plants. Gibberellic acid (GA3) is the most widely used plant growth regulator which increases stem elongation along with plant height and growth in various crops (Harrington et al., 1996, Akter et al., 2007; Emongor, 2007). Length of shoot is used as parameter to determine whether the plant is growing or not. Calculations are done from the base to the tip of the shoot. Ayala-Silva et al. (2005) showed that application of GA3 increased plant height in *Linum usitatissimum* L. GA3leads to increased vegetative growth of plants with vigorous shoots, and it has been revealed earlier for *Allium cepa* (Ud-Deen et al., 2005; Yamaguchi, 2008; Yu et al., 2009), *Colocasia antiquorum* (Ud-deen, 2009; Ouzounidou et al., 2011; Nagwa et al., 2013), in *Gladiolus* (Sarkar et al., 2014) and in *Polianthes tuberosa* (Sultana et al.,2016). Ilias et al. (2007) reported that shoot length was significantly enhanced in *Abelmoschus esculentus* by the GA3 foliar application. GA3 stimulated shoot length significantly in *Lagenaria siceraria* (Vwioko & Longe, 2009). Hussein (2009) reported that there is a significant increase in shoot length after foliar application of GA3in *Cryptostegia granndiflora* and the similar results were also observed by Shahin et al. (2010) in *Malus domestica* Bork. GA3promoted the length of the stem in *Allium cepa* (Shukla et al., 2010; Ouzounidou et al., 2011; Sisodia et al., 2012; Nagwa et al., 2013) and also regulate shoot length in *Solanum lycopersicum* (Choudhury et al., 2013). Savik and Guney, (2013) had revealed from their studies that after foliar application of 5000 mg/L of GA3 shoot length without branches was found to be 2.71 times greater than that of control group and 74% greater in total length of *Melissa officinalis* L. and similarly, by applying 1000 mg/L GA3, the length of stem is 87% greater than that of the control group in terms of length

of the stem without branches, and 38% greater in total length. Karimi et al. (2014) observed that GA3 at different concentrations stimulated stem to elongate more than control. Shoot length about 83.69 cm. was significantly increased after using 150 ppm of GA3 in *Gladiolus* (Sarkar et al., 2014). GA3 after exogenous foliar application had showed regulatory effect in *Capsicum annuum* (Pérez-Jiménez et al., 2015) and the shoot length of *Polianthes tuberose* found to be increased upto 53.59 cm at 200 ppm of GA3 after spraying 90 DAP (Sultana et al., 2016). Similar results have been reported by Alam and Kumar, (2017) in *Grewia subinequalis.* GA3 promote the growth of stem in *Stevia rebaudiana* (Yoneda et al., 2018).

It was also observed by Hala & Wafaa (2001) that foliar application of the plants with IAA at three concentrations (12.5, 25 and 50 ppm) induced increments of the plants height in *Vigna unguiculata.* Sarkar et al. (2002) stated that 100 ppm IAA produced the highest plant height (ie. 43% to than that of control plants) in *Glycine max* after 100 days of spraying. Plant height increased effectively by IAA application in *Vigna radiata* (Quaderi et al., 2006). Ashraf et al. (2006) found that IAA had regulatory effect in *Hordeum vulgare* L. to enhance the plant height as compared to control. El-Saeid et al. (2010) in their work on *Vigna unguiculata* plants for studying the growth characters under the influence of IAA reported that the higher concentration of IAA (100 mg/L) decreased shoot length of the plants about 8.90% as compared to the control plants. Foliar application of IAA at 50, 100 and 150 ppm, was found to increase the plant height in *Jatropha curcas* (Joshi et al., 2011).

Leaf area: Leaf is the place where the process of photosynthesis to construct the plant dry matter is occur. Leaf area is the result of leaf length, leaf width and leaf constant multiplication (Gardner, 1991). Leaf area on a plant is important to count because it affects the process of photosynthesis in plants. Leaf area was found to be affected in different ways after applying GA3 and IAA on various plants. *Brassica compestris* plants sprayed with 10^{-6} mol L^{-1} of GA3 showed 83.73% enhanced leaf area than that of untreated plants after 75 days of sowing (Khan et al., 2002). Khristyana et al. (2005) revealed that the 75 ppm concentration of GA3 in *Plantago* major showed significant increase with control. Ilias et al. (2007) also reported that leaf area was significantly enhanced by the foliar application of GA3. GA3 caused a significant decrease in leaf area in all stages of growth in *Cathranthus roseus* (Abdul Jaleel et al., 2008). Mukhtar (2008) reported that GA3 treatment increased the leaf area in *Hibiscus sabdariffa L.* Vamil et al. (2010) stated that the leaf area was found to be increased in *Bambusa arundinaceae* after exogenously foliar spray of GA3. Leaf area was increased in *Simarouba glauca* under foliar application of GA3 (Patil & Gaikwad, 2019). Increased leaf area by IAA treatment observed by Mishra et al. (2000) in *Dendrobium* and by Nandhini Devi

& Chezliyas 2001) in *Scadoxus multiflorus.* Spraying of 200 ppm of IAA on plants of *Verbascum Thapsus* reflected positive response towards maximum increase in leaf area about 84.12% than untreated plants (Bhandari et al., 2009). Khandaker et al. (2018) stated that application of IAA increased the leaf area of the *Abelmoschus esculentus*.

Number of leaves: It was reported in many studies that application of GA3and IAA enhanced number of leaves in various plants. Sarkar et al. (2002) observed that GA3treated *Glycine max* plants produced higher number of leaves. Tyagi et al. (2007) carried out an experiment to study the effect of GA3 and revealed that it increased the number of leaves per plant in *Allium cepa.* Mukhtar (2008) reported that 100 ppm of GA3 treatment increased about 26.03% leaf number in *Hibiscus sabdariffa L.* Patel et al. (2010a) suggested that the application of GA3 significantly increased the number of leaves per *Allium cepa* L. plant as compared to control. Singh et al. (2011) observed 69.96% increment in the number of leaves in *Grewia subinaequalis* with the spray of 100 ppm of GA3 than that of control plants. Ayyub et al. (2013) found that foliar application of GA3 boosted number of leaves in *Abelmoschus esculentus* L. Patil & Gaikwad (2019) reported that after the foliar application of GA3, number of leaves increased in *Simarouba glauca* DC. It has been reported that 200ppm of IAA application increased about 78.20% of leaf number in *Allium cepa* over untreated plants (Hye et al., 2002). Kokare et al. (2006) found that exogenous application of IAA enhanced the leaf area. Behera et al. (2006) also revealed that foliar application of IAA enhanced the number of leaves in *Sesamum indicum* L. Agrawal & Dikshit (2008) stated that application of IAA increased the number of leaves in *Pisum sativum.*

Biomass and productivity: Water content is based on the weight change between fresh and dried leaves. Application of plant growth regulators cause an increase in plant biomass which demonstrated their positive influence on the developmental stages that lead to higher productivity. There are number of studies that have been reported that fresh and dry weight of plants were enhanced after applying the different concentration GA_3 and IAA. The increased biomass by the application of GA_3 may be due to a rapid increase in cell division, cell enlargement (Mostafa and Abou-Alhamd, 2011).

Fresh weight of plant: Fresh weight is the weight recorded immediately after the plant is harvested. GA_3 and IAA showed the different effects on fresh weight of various plants in many of the studies. After treatment with Gibberellic acid, the fresh weight in *Catharanthus roseus* was slightly increased (Jaleel et al., 2008). The increase percent of fresh weight of *Gerbera jamesonii* after treatment with 50ppm of GA_3 was 89.02% over control after eleven

days of spraying (Danaee et al., 2011). GA_3 treated plants significantly promoted the fresh weight in *Allium cepa* as compared to the untreated plants (Ouzounidou et al., 2011). Tsiakaras et al. (2014) examined the effect of exogenous foliar application of GA_3 on *Lactuca sativa* and observed that fresh weight of the plant was increased. Govind et al. (2015) concluded that fresh weight of *Allium sativum* was increased after treatment with GA_3. It was reported by Martin et al. (2015) that fresh mass of *Capsicum annum* was increased when plants were treated with GA_3 (10 and 15 mgL^{-1}). Vomil et al. (2011) evaluated the effect of IAA on *Bambusa arundinacea* and observed that fresh weight of the *Bambusa arundinacea* plants was increased. Tayyaba et al. (2013) resulted that fresh weight of *Plantago ovata* L. was not affected by the application of IAA. Utami et al. (2018) reported from his findings that fresh weight of *Lemnaminor* showed no apparent growth-promoting effect by exogenous application of IAA. The exogenously applied IAA increased the fresh weight in *Medicago sativa* (Sosnowski et al., 2019).

Dry weight of plant: Dry weight is the weight recorded after drying the plant at temperature higher than the ambient air temperature. Dry weight of plant depends on the speed capability of cells to divide, enlarged and elongated. The speed of cell activity can be influenced by growth of hormones such as auxin and gibberellin. The addition of these growth hormones exogenously is expected to accelerate the growth process. Ibrahim et al. (2007) reported that the application of GA_3 significantly increased the total dryweight of *Vicia faba*. Akter et al. (2007) observed highest total drymatter was found with 50 ppm GA_3 (33.68 g) in *Brassica compestris* and the lowest total dry matter was obtained from the untreated plants (25.09 g). In this study the increase in percentage of total dry weight of plant after treatment with GA_3 (50ppm) was calculated about 74.50% over control plants. Marie et al. (2007) studied the effect of GA_3on *Abelmoschus esculentus* L. and observed an increase in the dryweight of plant as compared to control. GA_3slightly increased the dry weight in *Catharanthus roseus* (Jaleel et al., 2008). Plant dry mass found to be increased when *Capsicum annum* plants were treated with GA_3 (10 and 15 $mg{\cdot}L^{-1}$) (Martin & Christian, 2015). Spraying of GA_3 caused a significant increase in dry weight of *Fenugreek* (Madhoor & Faisal 2020). Application of IAA increased dryweight in *Lens culinaris* (Naeem et al., 2004). IAA treated plants exhibited higher values of dry weight in *Brassica* (Afroz et al., 2005). Shah (2011) reported that IAA application increased dry mass of *Nigella sativa*. Sadak et al. (2013) observed that after applying 75 mg/l of IAA, dryweight of plant *Viccia faba* was increased about 61.30% in comparison to untreated plants. Studies of Ogwu et al. (2015) on *Citrullus lanatus* revealed that IAA treatments have positive effects on dry weight of plant.

Relative water content: Relative water content is a most meaningful index for dehydration tolerance and considered a measure of plant water status and also reflecting the metabolic activity in tissue. Relative water content is a physiological indicator of available water content in favor of tissue metabolism (Yan et al., 2016; Zhao et al., 2017). Many of researchers have been resulted from their findings that relative water content was altered in many ways after treating plant with $GA_3$and IAA. Relative water content was found to be decreased after the foliar application of GA_3 in *Trigonella foenum graecum L*. in comparison to control plants (Modi et al., 2011). Pazoki et al. (2012) resulted from his findings that GA_3 increased relative leaf water contents in *Thymus vulgaris*. After the exogenous foliar application of GA_3 relative water content of *Triticum aestivum* was increased (Hamdia, 2013). Sayed & Gadallah (2013) also resulted that after treating with GA_3 relative water content was enhanced in *Helianthus annuus*. It has been reported that Relative water content of *Plantago coronopus* was increased after treatment with GA_3 (Bueno & Cordovilla, 2021). Jamwal et al. (2021) revealed that leaf relative water content was decreased about 105.27% in *Fagopyrum esculentum* after treatment with 50 ppm of GA_3 in comparison to control plants. Ashraf & Harris (2004) reported from his findings that foliar spray of IAA was effective in maintaining the leaf relative water content in *Hordeum vulgare. Zea mays* plants showed increased relative water content by exogenous foliar application of IAA (Kaya et al., 2010). After the exogenous foliar application of IAA, relative water content of *Triticum aestivum* was increased (Hamdia, 2013). Zayed et al. (2017) resulted from his studies that IAA application increased the relative water content in *Helianthus annuus* L. An increase in the relative water content was also detected in *Brassica juncea* of applying IAA exogenously on the leaves (Mir et al., 2020). Bueno & Cordovilla (2021) reported that relative water content of *Plantago coronopus* was increased about 92.41% after treatment with IAA in comparison to untreated plants.

Physiological aspects: Plant growth regulators are known to control plant's physiological processes and affect the physiological efficiency of plants including growth, photosynthesis and accumulation of assimilates. They are readily absorbed and move rapidly through tissues when applied to different parts of the plant. Plant growth regulators used in small concentrations, modify or regulate physiological processes in an appreciable measure in plants. Hamdia, (2013) reported that GA_3 or IAA increase the soluble sugars, soluble protein and amino acid. IAA also increases total carbohydrate, free amino acids and total phenolic content (Sadak et al., 2013).

Membrane content: The membrane stability index measures the percentage of damage and describes the abilityofthe membrane to survive in the drought stress. Water stress results changes in cell membrane permeability (Almeselmani et al., 2011). Memrane stability index was positively enhanced after the exogenous foliar spray of GA_3 and IAA on different plants. GA_3at different concentrations improved membrane stability index in *Gladiolus* (Singh et al., 2008). Danaee et al. (2011) reported from his studies that exogenously applied 50ppm of GA_3 increase membrane stability index about 88.36% in *Gerbera jamesonii* over control. GA_3 are known to increase membrane permeability in plant cells of *Emblica officinalis* Gaertn (Singh & Singh, 2015). Rady et al. (2021) reported from his findings that memberane stability index was positively increased in *Vicia faba* L. after the exogenous foliar application of GA_3. IAA treatment increased membrane stability index significantly in *Helianthus annus* plants (Singh et al., 2011). Zayed et al. (2017) resulted from his studies that IAA application increased the membrane stability in *Helianthus annuus* L. After the exogenous foliar spray of IAA membrne statability index of *Brassica napus* L. was enhanced (Aslam et al., 2021).

Carbohydrate content: In plants carbohydrate is produced by photosynthesis. They are well known for their essential role as vital sources of energy and carbon skeletons for organic compounds and storage components. They perform numerous roles in plants having the ability to regulate the lipid metabolism by lowering of triglycerides and cholesterol (Collins et al. 2016; Ganesan & Xu 2019). Carbohydrate content was found to be increased in various plants after treating exogenously by the foliar spray of IAA and GA_3. Abu-Grab Ebrahim (2000) investigated the highest carbohydrates content in *Allium cepa* after the exogenous foliar spray of GA_3. The application of GA_3 increased the plant growth by improving carbohydrate metabolism in *Lycopersicon tuberosum* (L.) (Salimi et al., 2010). Accumulation of carbohydrate content was observed after the foliar application of GA_3 in *Trachyspermum ammi* L by Rohamare et al. (2013). Exogenously applied IAA enhanced the total carbohydrate contents in *Vicia faba* L. Ogwu et al. (2015) in *Citrullus lanatus* revealed that IAA treatments has positive effects on total carbohydrate contents of the plant. IAA enhanced the carbohydrate content in *Lens culinaris* (Akhtar et al., 2012). It has been resulted from the findings of El-Awadit et al. (2017) that IAA enhanced the carbohydrate content in *Cicer arietinum.* Total carbohydrate contents of the *Viccia faba* cultivars were significantly increased in response to different concentrations of GA_3 and IAA treatments as compared with control plant (Refaat & Saber, 2016).

Total amino acid: Amino acids are essential in the synthesis of proteins and precursors in the

formation of secondary metabolites (Perez & Garcia, 2009). They participate in protein phosphorylation, synthesis of hormones and antioxidant capacity (Wu, 2009). Plant growth regulators (IAA and GA_3) positively enhanced the amino acid content in many of plants. Exogenous application of GA_3 increase total amino acids in *Triticum aestivum* (Hamdia & Abdel, 2013). Hamdia (2013) reported from his findings that amino acid content was enhanced in GA_3 treated *Triticum aestivum* plants. Refaat & Saber (2016) stated that total amino acid in protein of *Vicia faba* seeds was positively enhanced after treatment with GA_3. Hamdia (2013) reported from his findings that amino acid content was enhanced in IAA treated *Triticum aestivum* plants. IAA recorded an increase in the content of total amino acid in protein of *Vicia faba* seeds (Refaat & Saber, 2016). Amino-acid contents increased with an increase in IAA concentrations in *Spinacia oleracea* (Gong et al., 2019). Rahman et al. (2019) reported that amino acid was increased after the exogenous application of IAA on *Vigna radiate.* Abdel Latef et al. (2021) resulted an increase in total amino acid contents in *Vicia faba L*. plants after treating exogenously with different concentration of IAA. Exogenous application ofIAA decreased amino acids in *Cynara cardunculuys var. scolymus* (Dawood et al., 2021).

Protein content: Proteins play a vital role in every aspect of life including metabolic activities as catalysts, transporters, and messengers in handling systems of living cell Dhabhai & Batra, 2011; Escobar, 2001). GA_3 and IAA positively increased the protein content in number of plants. Exogenously applied GA_3 induced a marked accumulation of protein content in *Hibiscus sabdariffa* (Abdel-Latef, 2003; Abou Al-Hamd, 2007). GA_3 induced a marked accumulation of protein content in *Sorghum* (Azooz et al., 2004). Vani et al. (2004) studied the effect ofGA_3 on *Zea mays* and noted a significant increase in protein content. Shah (2007) observed increase in the protein content of *Nigella sativa* under the influence of GA_3. GA_3 enhanced the protein content in *Balanites aegyptiaca* plants (Mostafa & Abou Alhamd, 2011). Increased protein content was observed in exogenously GA_3treated *Cassia angustifolia* (Shitole & Dhumal, 2014). Borse et al. (2000) noted significant enhancement in proteins due to the effect of IAA in *Solanum khasianum*. The positive influence of IAA on protein content was reported in *Pennisetum glaucum* by (Kumar et al., 2001). IAA application increased the protein content in *Pleurotussajor-caju* (Mukhopadhyay et al., 2005). Kumar et al. (2005) resulted that after treating with IAA protein content was increased in *Zea mays*. Protein content was increased by the exogenously applied IAA on *Ziziphus mauritiana* (Gadil et al., 2006). Increase in proteins due to IAA treatment in *Vigna mungo* was recorded by (Sritharan et al., 2005). The protein content was increased in *Vigna radiata* L. after applying IAA

exogenously on the leaves (Mohammed, 2007). Shah (2011) reported that IAA increased the protein content of the plant when applied exgoneously on the leaves of *Nigella sativa*. Choudhary et al. (2013) stated that foliar spray of IAA had showed the positive effect on protein content in *Viccia faba*. Soluble protein content increased with an increase in IAA concentration in *Spinacia oleracea* (Gong et al., 2019). IAA treatment caused a significant increase in protein content in *Phaseolus vulgaris* L. (Ismail et al., 2021).

Proline content: Proline is the most important amino acids that accumulate in various tissues of the plant, particularly in the Leaves. It is a proteinogenic amino acid for primary metabolism (Szabados & Savoure, 2010; Gupta et al., 2015). Proline content is variably affected by GA_3 and IAA in different plants. Darwesh & Mohamed (2009) found that foliar application of GA_3 increased proline in *Phoenix dactylifera.* Exogenous foliar application of GA_3 increased proline content in *Phaseolus mungo* (Guru et al., 2012). Dheeba et al. (2015) resulted that exogenous foliar application of GA_3decreased the level of proline content in *Vigna mungo.* Al-harthi et al. (2021) reported that exogenous application of GA_3 decreases the level of proline content in *Cucurbita pepo*. Sadak et al. (2013) in *Viccia faba* and Ogwu et al. (2015) in *Citrullus lanatus* reported that exogenous foliar application of IAA resulted the positive effects on proline contents of the plants. The application of IAA on the leaves of *Pisum sativum* increased the proline content as compared to untreated plants (Sergiev et al., 2017). IAA increased the proline content in *Brassica juncea* plants (Mir et al., 2020).

Biochemical aspects: Plant growth regulators play a very important role in various biochemical processes in plants and so that considered to be an effective tool for increasing crop yields. The plant regulator GA_3 was found to be very effective on production of photosynthetic pigments in many of the studies. The influence plant growth regulators in comparison to control on biochemical parameters, namely ascorbic acid, tocopherol, phenol, flavonoid, chlorophyll, stevioside and rebaudioside A is dicussed below.

Ascorbic acid: Ascorbic acid possesses powerful antioxidant activity by enhancing redox reactions in cells thereby contributing to non-enzymatic transport of hydrogen to the plant tissues (Georgeseu, et al., 2019). It also neutralises peroxide radicals whilst promoting enzymatic activity in plant tissues (Logan, 2005; Al-Majidi & Al-Qubury, 2016). The concentration of ascorbic acid was recorded maximum in many plants after treating with $GA_3$and IAA. The maximum ascorbic acid content (670 mg/100 g fruit pulp) was noted with the application of 50 ppm GA_3 in *Emblica officinalis* Gaertn (Singh & Singh, 2015). In *Psidium guajava* L. the highest ascorbic acid content (135.30mg/100g) was reported at 50

ppm GA_3 whereas lowest content (64.27mg/100g) was found in control plants (Lal & Das, 2017). Singh et al. (2017) reported that ascorbic acid was increased in *Grewia subinaequalis* DC by the application of GA_3. The ascorbic acid was significantly increased in the *Mangifera indica* L. after the treatment with GA_3 in comparison to untreated plants (Singh et al., 2017). Maximum ascorbic acid contentin *Grewia asiatica* fruits (44.25 mg/100gm of pulp) was observed at 100ppm GA_3 (Kaur, 2020). The accumulation of ascorbic acid is lower in GA_3-treated *Ocimum Gratissimum* L. explants (Olaitan, et al., 2022). Harinder & Anis (2020) reported that ascorbic acid increased in the IAA treated *carica papaya* plants in comparison to control plants. IAA enhanced ascorbic acid in *Catharanthus roseus* (Sidkey, 2020).

Toccopherol: Tocopherols are lipophilic antioxidants that are synthesized exclusively in photosynthetic organisms. Usually, leaves commonly accumulate α-tocopherol, whereas seeds are rich in γ-tocopherol. GA3 and IAA reflected positive effect on the toccopherol content of various plants. Jaleel et al. (2007) reported that tocopherol significantly increased in *Catharanthus roseus* after spraying GA3 exogenously. The toccopherol content in the GA3treated plants of *Cucurbita pepo* L. was found higher in comparison to control plants (Sedghi et al., 2008). Tocopherol content of *Cannabis* was decreased in 50 µm GA3 treatment and increased in 100 µm GA3treatment (Mansouri et al., 2011). Rady et al. (2021) reported from his findings that toccopherol content was increased in *Vicia faba* L. after treatment with GA3. Jaleel et al. (2009) stated from their studies that toccoperol content was in significantly increased after applying IAA exogenously on the leaves of *Catharanthus roseus* L. By the foliar application of IAA, toccoperol content was positively enhanced in *Catharanthus roseus* (Sidkey, 2020).

Phenolic content: Natural phenolic compounds are plant secondary metabolites that hold an aromatic ring bearing at least one hydroxyl group (Tungmunnithum et al., 2018). These are important plant constituents with redox properties responsible for antioxidant activity (Soobrattee et al., 2005). These secondary metabolites are derived from the pentose phosphate, shikimate, and phenyl propanoid pathways in plants (Randhir and Lin, 2004). Treatment of GA3 decreased phenolic compounds in *Solanum lycopersicum* (Araby et al., 2006). *Salvia miltiorrhiza* plants when treated with GA3 showed a significant increase in phenolic compounds (Liang et al., 2013). Ali et al. (2015) reported that a significant increase was observed in *Artemisia absinthium* L. after exogenous foliar application of GA3. GA3 enhanced phenols accumulation in *Catharanthus roseus* (Sidkey, 2020). A significant increase in phenolic compounds was reported in GA3-treated *Zea mays* L. plants (Shahzad et al.,

2021). Kadiogul & Atalay (2002) noted decrease in phenolic content due to the treatments of IAA in *Diospyros lotus.* Naguib et al. (2003) mentioned that total phenolic compounds of the *Catharanthus roseus* plants were significantly increased due to the foliar application of IAA (50 and 100 ppm) compared with control plants. Low concentration of IAA promoted the production of phenolic compounds in the hairy roots of *Panax ginseng* (Jeon et al., 2007). From the studies of Sadak et al. (2013) and Ogwu et al. (2015) it has been revealed that IAA treatments has positive influence on total phenolic contents in *Viccia faba* and *Citrullus lanatus*, respectively.

Flavanoid: Flavonoid compounds are widespread in plant kingdom where they act as antioxidants and free radical scavengers. These are the subclass of anthocyanins, (Tural & Koca, 2008) and play a vital role in the physiology of plants by producing the red and purple anthocyanin pigments (Taylor & Grotewold, 2005). The highest content of flavanoid was recorded in GA3 treated fruits of *Syzygium samarangenese* (Khandaker et al., 2013). Sakya et al. (2022) reported the highest content of flavonoid in GA3 treated *Medinilla ver rucosa.* The exogenous supply of IAA led to an increase in the production of flavonoids in the adventitious roots of *Hypericum perforatum L.* (Cui et al., 2010). Chang et al. (2017) reported that IAA had significantly increased the flavanoid content in *Fagopyrum esculentum* Moench. Flavnoid content increased in *Chrysanthemum indicum* after the spraying of IAA (Ghimire et al., 2022).

Chlorophyll content: Chlorophyll is the main pigment of plant photosynthesis and plays an important role in different physiological processes of plants (Gu et al., 2016). Gibberellic acid has stimulatory effects on chlorophyll content (Harrington et al., 1996). Chlorophyll content was found to be highest after the foliar application of GA3in *Codiaeum variegatum* plants (Eid & Abou-Leila, 2006). Shah (2007) reported that exogenous foliar application of GA3enhanced chlorophyll content in *Brassica compestris* and in *Hibiscus sabdariffa* L. (Mukhtar, 2008). Ayyub et al. (2013) found that foliar application of GA3boosted chlorophyll content in *Abelmoschus esculentus* L. Moench. Highest (26.04%) chlorophyll content was observed after the exogenosly foliar application of 100 mg L^{-1} GA3in *Trachyspermum ammi* L. (Rohamare et al., 2013). It has also been reported that the foliar application of GA3 increased leaf chlorophyll content in *Mentha arvensis* L. (Bose et al., 2013) and also in *Vaccinium virgatum* (Zang et al., 2016). Atteya et al. (2018) confirmed that GA3 enhanced chlorophyll content of *Simmondsia chinensis* plant. IAA exerts positive influence on plant growth by enhancing chlorophyll content in *Lens Culinaris* Medik (Naeem et al., 2004). Vamil et al. (2010) observed the positive effects of IAA on chlorophyll content of *Bambusa*

arundinaceae. Kaya et al. (2013) revealed the positive effect of IAA on chlorophyll content of *Zea mays*. The studies of Sadak et al. (2013) on *Viccia faba* and Ogwu et al. (2015) on *Citrulluslanatus* revealed that IAA treatments has positive effects on chlorophyll a and b contents of the plant. It was also observed that a lower concentration of IAA increased the chlorophyll content in *Abelmoschus esculentus* L. Moench (Khandaker et al., 2018). Hanaa & Safaa (2019) concluded that foliar application of IAA (100 ppm) can enhance the chlorophyll content of *Triticum aestivum* L.

RESEARCH GAP IDENTIFICATION

Review of literature reveals that many studies have been conducted on the effect of plant growth regulators on various medicinal plants but very little work has been done on the effect of phytohormones (IAA and GA_3) on *Stevia rebaudiana* plant. Exogenous application of plant growth regulators is a strategy to increase yield, improve crop quality and accumulation of secondary metabolites in plants. The steviol glycosides obtained from stevia are considered to be the best alternative source of sugar especially for diabetic patients. Globally, food and pharma industries have recognized the importance of zero calorie stevia extract because of it is a natural source and its health benefits appeals to many consumers. Therefore, there is need to conduct research work on the following aspects of *Stevia rebaudina* Bertoni:

- Study the effect of plant growth regulators (IAA and GA_3) on the morphological traits of the plant.
- Impact of phytohormones (IAA and GA_3) on the physiological parameters of the plants.
- Influence of plant growth regulators (IAA and GA_3) on the biochemical parameters of *Stevia rebaudiana* Bertoni.

OBJECTIVES

The objectives of study of effect of phytohormones on the *Stevia rebaudiana* Bertoni are to:

1. Determine IAA and GA_3 induced morphological changes.
2. Study the effect of IAA and GA_3 on biomass production and physiological parameters.
3. Evaluate the effect of IAA and GA_3 on biochemical parameters.

PROPOSED METHODOLOGY

Seedlings of *Stevia rebaudiana* Bertoni will be purchased from Institute of Himalayan Bioresource Technology, Council of Scientific Industrial Research (CSIR), Palampur, Himachal Pradesh, India. Experiments will be conducted in the laboratory of Department of Bio Sciences, Career Point University, Hamirpur (76º-17’50” to 76º-43’-42” eastern

longitudes and 31º-24'-48" to 31º-53'-35" northern latitudes), Himachal Pradesh, India (Ram chand et al., 2017).

Experimental setup: Two months old plants of *Stevia rebaudiana* Bertoni will be transferred to the plastic pots of size (21 cm diameter and 19 cm height) in the month of June, 2023. These pots will be filled with 6 kg mixture of the air dried local sandy soil, organic manure and sand in the ratio of 3:2:1 respectively. Plants will be left for adaptation for about 15 days. The plants will be protected from the rain by using polythene cover. The polythene cover will be pulled back immediately after the rain so that the plants receive maximum sunlight. To avoid the position effect on the plants, the position of the pots will be changed weekly. Thereafter, solutions of these two plant growth regulators i.e., IAA and GA_3 each at 25 mg/L, 50 mg/L and 100 mg/L concentrations will be exogenously sprayed on the leaves and stems of the 60 days old plants by using hand sprayer. Each plant will be sprayed with 20 ml of the various concentrations after the intervals of 10 days for 180 days. For each concentration twelve replicates will be used. Control plants will be sprayed with distilled water. Sampling will be done after 60, 120 and 180 days of spraying. Leaves will be used for analysis of the various growth parameters.

Physiochemical properties of soil: For soil analysis, viz., pH, organic carbon, available N, P, K and Bulk density, composite soil samples will be collected from 0-20 cm and 20-40 cm depth from each plot, before transplanting and after harvesting of crops, air dried and sieved to pass a 2mm screen. The pH of soil will be determined using (1:2.5) of soil and water suspension respectively (Jackson, 1973). Organic carbon, available nitrogen, phosphorus and potassium will be determined by using rapid titration method (Walkley & Black, 1934); alkaline potassium per manganate method (Subbiah & Asija, 1956); neutral 1N ammonium acetate method (Merwin & Peech, 1951), respectively. Whereas, Bulk density (g cm^3) will be estimated by the specific gravity method (Singh, 1980

Morphological Aspects:

Shoot Length: Shoot length will be measured from the base of the stem up to the growing tip of the main stem by using a scale in centimeter (cm).

Root Length: Root length of plant will be measured from the base of the root up to the growing tip of the main root by using a scale in centimeter (cm).

Leaf Area: Leaf area of mature leaf will be calculated by using formula AL = 0.73 (LL×LW) (Musyimi et al 2010). It will be measured in (cm^2).

$$AL=0.73\ (LL\times WL)$$

Where, LL is the leaf length and WL is the maximum width measured for each leaf.

Number of leaves: Number of fully extended mature leaves on the main stem and branches will be counted and recorded.

Biomass and production:

Fresh weight of plant: Plants will up-rooted and their fresh weight will be recorded in grams (g) by using electronic balance.

Dry weight of plant: The sample will be dried in an oven for 48 hours at 72°C. Dry weight of the sample was weighed using an electronic balance in grams (g) (Kacar, 1972).

Relative water content: The fresh weight of leaves from each treatment will be recorded. The leaves will be the immersed in distilled water in beakers and left for 24h. Thereafter, fully turgid leaves will be weighed again. The leaves will be dried in oven for 72h at 70°C, until constant weight of leaves will be obtained. Relative water content (RWC) of leaves will be calculated according to Wheatherley (1950). Relative water can be calculated by the equation:

$$\mathrm{RWC} = \frac{\text{Fresh mass} - \text{dry mass}}{\text{Saturated mass} - \text{dry mass}} \times 100$$

Physiological Aspects:

Membrane stability index: The leaf membrane thermostability index (MSI) was measured following the protocol of Premachandra et al. (1990) as modified by Sainnan (1994). For determination of membrane stability index of leaf, 2 mg fresh leaf of each treatment and 20 ml double distilled water were taken in a Falcon tube. The Falcon tube was then placed in the water bath for boiling at 40 °C for 30 min. After that, the electrical conductivity (C_1) of this boiling sample was measured by an electrical conductivity meter. Consequently, another sample of leaves of the same plant was placed in a water bath for boiling at 100 °C for 10 min and electrical conductivity was recorded in the same way (C_2). Finally, MSI was estimated with the following equation:

$$\mathrm{MSI} = 1 - \frac{\mathrm{C1}}{\mathrm{C2}} * 100$$

Total carbohydrates: Total carbohydrates will be determined in plant tissue as described by Hedge and Hofreiter (1962) through anthrone method. Weigh 100 mg of the sample into a boiling tube. Hydrolyze by keeping it in boiling water bath for 3 hours with 5 mL of 2.5N HCl and cool at room temperature. Neutralize, it with solid sodium carbonate until the effervescence ceases. Make the volume to 100 ml and centrifuge at 10,000 rpm for 5 minutes. Collect the supernatant and took 0.5 and 1ml aliquots for analysis. Prepare the standards by

taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard. Zero serves as blank. Make the volume to 1ml in all the tubes including the sample tubes by adding distilled water. Then, add 4 ml of anthrone reagent. Heat for eight minutes in a boiling water bath. Cool rapidly and read the green to dark green color at 630nm. Draw a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis. From the graph calculate the amount of carbohydrate present in the sample tube.

$$\text{Amount of carbohydrate present in 100mg of the sample} = \frac{(\text{mg of glucose})}{(\text{Volume of test sample})} \times 100$$

Total amino acids: Total amino acids were determined according to the method of Moore and Stein (1948). Weigh 500 mg of plant sample and grind it in a pestle and mortar with a small quantity of acid-washed sand. To this homogenate, add 5to 10 ml of 80% ethanol. Centrifuge and save the supernatant. Repeat the extraction twice with the residue and pool all the supernatants. Reduce the volume if need by evaporation and use the extract for the quantitative estimation of total free amino acids. To 0.1 ml of extract, add 1 ml of ninhydrin solution. Make the volume to 2ml with distilled water. Heat the tube in boiling water bath for 20 min. Add 5 ml of the diluents and mix the contents. After 15 min read the intensity of the purple color against a reagent blank in a colorimeter at 570 nm. The color is stable for 1h. Prepare the reagent blank as above by taking 0.1 ml of 80% ethanol instead of the extract. For the preparation of standard, dissolve 50 mg leucine in 50 ml of distilled water in a volumetric flask. Take 10 ml of this stock standard and dilute to 100 ml in another flask for working standard solution. A series of volume from 0.1 to 1ml of this standard solution give a concentration range 10 to 100mg. Proceed as that of the sample and read the color. Draw a standard curve using absorbance versus concentration. Determine the concentration of the total free amino acids in the sample and express as percentage equivalent of leucine.

Protein: Protein content will be determined by the method given by Lowry et al. (1951). Leaf sample (500 mg) will be grind by pestle and mortar in 5-10 percent of the phosphate buffer. After centrifugation supernatant will be used for protein estimation. Pipette out 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard into a series of test tube. Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes. In all the test tubes, make the volume to 1 ml. A tube with 1 ml of water will be served as the blank. 5 ml of reagent C (alkaline copper solution) will be added to each tube including the blank. Properly mix the contents and allow it to stand for 10 minutes. After that, 0.5 ml of reagent D (Folin-Ciocalteau Reagent) will be added, mix the contents properly and incubate at room temperature in the dark for 30 minutes.

Blue color will be appeared. Reading will be taken at 660 nm. A standard graph using Bovine Serum Albumin will be plotted and amount of protein in the sample will be calculated. The amount of protein present in the sample was expressed (in mg/gm) as follows:

$$\text{Amount of protein present in sample} = \frac{\text{mg of protein}}{\text{Volume of test standard}} \text{Conc. of Standard}$$

Proline: The proline content will be determined by using acid ninhydrin reagent by the method of Bates et al. (1973). Homogenize a grounded leaf sample (200 mg) in liquid nitrogen with 4 ml of 3% sulphosalicylic acid and centrifuge the homogenate at 3000rpm for 10 minutes. Mix 2 ml of the filtrate with 2 ml acid ninhydrin and 2 ml of glacial acetic acid in the test tube. Keep the test tube in water bath at 100 °C for one hour and then place on ice to cool down. Add 4 ml of toluene to the reaction mixture and stirr vigorously using vortex for 15 to 20 seconds. Two seperate layers will be formed and take out the layer containing toluene and warm to room temperature. Read the intensity of the colour at 520 nm using toluene as a blank. Calculate the content as

$$\text{Amount of Proline} = \frac{\text{x}}{2} \times \frac{10}{250} \times 1000$$

Amount of proline present in the sample is expressed in µg/g.

Biochemical aspects

Ascorbic acid: Ascorbic acid concentration will be determined according to the method described by Roe & Keuther, (1943). Firstly, ascorbic acid will be extracted from 100 mg of the plant sample using 10 mL of 4 % trichloroacetic acid. The supernatant will be obtained after centrifugation at 2000 rpm for 10 min will be treated with a pinch of activated charcoal and will be shaken vigorously using a cyclomixer and kept for 5 min. The charcoal particles will be removed by centrifugation and the supernatant will be used for the estimation of ascorbic acid concentration. Briefly, standard ascorbate (0.5 mL) ranging between 0.2 mM – 1.0 mM and 1.0 mL of the extract will be taken. The volume will be made up to 2.0 mL with 4% trichloroacetic acid. Dinitrophenyl hydrazine (DNPH) reagent (0.5 mL) will be added to all the tubes, followed by two drops of 10% thiourea solution. The contents will be mixed and incubated at 37 °C for 3 h in a water bath resulting in the formation of osazone crystals, which will be subsequently dissolved in 2.5 mL of 85 % sulphuric acid. Similarly, to the blank, DNPH reagent and thiourea will be added after the addition of sulphuric acid. The tubes will be cooled in ice and absorbance will be read at 540 nm in a spectrophotometer. A standard calibration graph will be constructed from where the concentration of ascorbic acid in the samples will be determined and will be expressed as mg/g of sample.

Tocopherol: The tocopherol content in the plant samples will be estimated spectrophotometrically by the method reported by Ranganna (1997). The plant samples (500 mg) will be homogenized in a 10 ml of 0.1N sulphuric acid and the volume will be finally made up to 50 ml by adding 0.1N sulphuric acid slowly, without shaking and the contents will be allowed to stand overnight. The contents of the flask will be shaken vigorously on the next day and filtered through Whatman No.1 filter paper. Aliquots of the filtrate will be used for the estimation of toccopheol. The plant extract, standard and water of 1.5 ml will be pipetted out into three centrifuge tubes namely test, standard and blank respectively. To all the tubes, 1.5 ml each of ethanol and xylene will be added, mixed well and centrifuged. After centrifugation, the xylene layer will be transferred into another tube. To 1.0 ml of xylene layer, 1.0 ml of 2, 2 ′- dipyridyl reagent will be added, stoppered and mixed. This reaction mixture will be taken in the spectrophotometric cuvettes and the extinctions of the test and the standard will be read against the blank at 460 nm. Then, in turn, beginning with the blank, 0.33 ml of ferric chloride solution will be added, mixed well and after exactly 15 minutes, the test and the standard will be read against the blank at 520 nm. The levels of tocopherol will be calculated using the formula:

$$\text{Toccopherol } (\mu g) = \frac{\text{A520} - \text{A460}}{\text{Std A520}} \times 0.29 \times 15 \times \frac{\text{Total volume of homogenate}}{\text{Volume used} \times \text{weight of tissue}}$$

Flavanoid: Total flavonoid content will be determined by Aluminium chloride method using quercetin as a standard (Olajire & Azeez, 2011). One ml of test sample and 4 ml of water will be added to a volumetric flask. Add 0.3 ml of 5 % Sodium nitrite, 0.3 ml of 10% Aluminium chloride will be added after 5 minutes. After 6 minutes of incubation at room temperature, 1ml of 1 M Sodium hydroxide will be added to the reaction mixture. Immediately the final volume was make upto 10 ml with distilled water. Absorbance of sample will be measured against the blank at 510 nm using a spectrophotometer. All the experiment will be repeated three times for precision and values will be expressed in mean ± standard deviation in terms flavonoid content (Quercetin equivalent, QE) per g of dry weight.

$$\text{Flavanoid } (\%) = \frac{\text{Weight of sample}}{\text{Weight of original sample}} \times 1 \times 100$$

Phenol: Phenol content will be estimated by Malick and Singh (1980). Weigh 500 mg of the sample and grind it with a pestle and mortar in 10 time volume of 80% ethanol. Centrifuge the homogenate at 10,000 rpm for 20 min. Save the supernatant. Re-extract the residue with 5 times the volume of 80% ethanol, centrifuge and pool the supernatants. Evaporate the supernatant to dryness. Dissolve the residue in 5 ml of distilled water (5 ml). Pipette out

different aliquots (0.2 to 2 ml) into test tubes. Make the volume in each tube to 3 ml with water. Add 0.5 ml of Folin-Ciocalteau reagent. After 3 min, added 2 ml of 20% Na2CO3 solution to each tube. Mix thoroughly. Place the tubes in a boiling water bath for exactly one min, cool and measure the absorbance at 650 nm against a reagent blank. Prepare a standard curve using different concentrations of gallic acid. From the standard curve the concentration of phenols in the test sample was determined and expressed as mg phenols/100g material.

The total phenolic content will be determine by using the formula as follows:

$C = C1 \times V/m$

Where, C = total phenolic content in mg/g, in GAE (Gallic acid equivalent),

C1 = concentration of Gallic acid established from the calibration curve in mg/ml,

V= volume of extract in ml, and

m = the weight of the plant extract in g.

Chlorophyll content: Cholorophyll extraction will be done by using dimethyl sulphoxide (DMSO) chlorophyll extraction technique of Hiscox and Israelstam (1979). For the extractions, glass centrifuge vials containing 7 ml DMSO will be preheated to 65°C in a water bath. Chlorophyll will be extracted from 100 mg fresh weight of each leaf sample. When the extractions will complete, samples will be removed from the water bath and each graduated vial will be topped up to 10 ml with DMSO using a Pasteur pipette; 3 ml of each extract will be then transferred to disposable polystyrene cuvettes. The spectrophotometer will be calibrated to zero absorbance using a blank of pure DMSO. Absorbance of both blank and sample will be measured at 645 and 663 nm. The elapsed time between removal from the water bath and completion of spectrophotometer measurements will be in the order of 20 min. Hiscox and Israelstam (1979) recommended the use of Arnon's (1949) equations:

Chla ($g\ l^{-1}$) = 0.0127 A663 – 0.00269 A645;

Chlb ($g\ l^{-1}$) = 0.0229 A645– 0.00468 A663;

Total Chl (gl^{-1}) = 0.0202A645+0.00802A663

The Chlorophyll (Chl) concentration of the extract calculated from these equations was then converted to leaf Chl content (mg Chl cm^{-2} leaf area)

Statistical analysis: All data will be analysed by two way analysis by of variance (ANOVA) by using Graph Pad Prism 5.2. For checking the significance difference between treatments, the mean of all values will be compared by Bonferroni test. Least significant difference (LSD) will be considered and 'F' test will be applied to evaluate the significance of data at 5% level of probability ($p<0.05$). Standard error will be drawn in all the graphics.

THESIS OUTLINE:

Chapter1	**Introduction**	This chapter will give the background of the Research topic.
Chapter2	**Literature Review, Research Gap and Objectives**	In this chapter, the major studies that have been published in research papers and the various methodologies used, will be summarized. Differences will be drawn from these studies and explanations that how research papers contribute to the literature. Research objectives have been decided on the bases of research gap.
Chapter3	**Methodology**	In this chapter, complete details of all the methods, Procedure and techniques will be provided.
Chapter4	**Result and Discussion**	In this chapter, results of the research work will be shown and compared with the previous findings.
Chapter5	**Summary and Conclusion**	In this chapter, the summary of results and future scopes of the further research will be provided.
Chapter6	**References**	References will be provided in Harvard reference system.

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CERTIFICATE
(SYNOPSIS APPROVAL CERTIFICATE)

This is to certify that the Synopsis for Ph.D. Thesis titled, **"Plant growth regulators mediated consequences on morpho-physiological and biochemical characteristics of *Stevia rebaudiana* Bertoni"**, submitted by **Ms. Sweta** to the Career Point University, Hamirpur (H.P.), India in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in the Division of Botany, Department of Bio Sciences, School of Basic and Applied Sciences has been approved by the Research Advisory Committee (RAC) after an Oral Examination of the student on dated 12/06/2023.

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